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Real-Time Characterization of Virulence Factor Expression in *Yersinia pestis* Using a Green Fluorescent Protein Reporter System

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Abstract

A real-time reporter system was developed to monitor the thermal induction of virulence factors in *Yersinia pestis*. The reporter system consists of a plasmid in *Y. pestis* in which the expression of green fluorescent protein (GFP) is under the control of the promoters for six virulence factors, *yopE*, *sycE*, *yopK*, *yopT*, *yscN*, and *lcrE*/*yopN*, which are all components of the Type III secretion virulence mechanism of *Y. pestis*. Induction of the expression of these genes *in vivo* was determined by the increase in fluorescence intensity of GFP in real time. Basal expression levels observed for the *Y. pestis* promoters, expressed as percentages of the positive control with GFP under the control of the *lac* promoter, were: *yopE* (15%), *sycE* (15%), *yopK* (13%), *yopT* (4%), *lcrE* (3.3%) and *yscN* (0.8%). The *yopE* reporter showed the strongest gene induction following temperature transition from 26ºC to 37ºC. The induction levels of the other virulence factors, expressed as percentages of *yopE* induction, were: *yopK* (57%), *sycE* (9%), *yscN* (3%), *lcrE* (3%), and *yopT* (2%). The thermal induction of each of these promoter fusions was repressed by calcium, and the ratios of the initial rates of thermal induction without calcium supplementation compared to the rate with calcium supplementation were: *yopE* (11 fold), *yscN* (7 fold), *yopK* (6 fold), *lcrE* (3 fold), *yopT* (2 fold), and *sycE* (2 fold). This work demonstrates a novel approach to quantify gene induction and provides a method to rapidly determine the effects of external stimuli on expression of *Y. pestis* virulence factors in real time, in living cells.

Introduction

The ability to measure changes in gene expression using real-time reporter systems has provided unprecedented insight into expression regulatory elements of biological systems. It is now possible to determine the effect of changes in environmental variables such as nutrient concentration, pH, temperature, and other external stimuli on the expression of genes of interest *in vivo* without disturbing the system. Reporter systems also reveal the combined effects of translation as well as transcription, and thus represent the effects of all control elements on protein production. As such, expression levels obtained from reporter systems are distinct from microarray studies, where the quantifiable signal depends on the transcriptional event. Reporter systems therefore provide a better representation of the dynamics of the biological system under study.

Of the reporter systems currently in use, green fluorescent protein (GFP) [1] has unique properties that make it particularly well suited for real-time studies. The gene for GFP can be incorporated into a plasmid vector, whereby the protein can be functionally expressed in a wide variety of organisms, and the protein is quite stable to changes in ionic strength, pH and temperature. Further, GFP does not require the addition of any enzymatic cofactor, though it does require stoichiometric amounts of oxygen, and sufficient time to develop the fluorophore.

The focus of this real-time expression study using a GFP reporter is the Type III secretion virulence mechanism of *Yersinia pestis*. *Y. pestis* is a non-motile, Gram-negative bacterium and the etiological agent of plague [2]. Normally, *Y. pestis* is found in two natural reservoirs, fleas and mammalian hosts, and is easily transferred between these reservoirs via fleabite. When the

pathogen senses this change in environment, gene expression is coordinated to ensure pathogen survival in the new host. Significantly, the change in physiological environment from flea vector to mammalian host can be modeled *in vitro* by adjusting the temperature from that of the flea, 26ºC, to that of the mammalian host, 37ºC. [3] Under host physiological conditions, a notable change in gene expression is the up-regulation of a Type III secretion system and other virulence factors encoded on the low-calcium response plasmid, pCD1. [4] Better characterization of the expression of virulence factors as a function of host environment will further our understanding of the virulence mechanism of *Y. pestis*.

Previously, reporter systems have been used to study gene expression in *Yersinia*. For example, the expression levels of *yopK* [5] and *yopE* [6] in *Y. pestis* were studied using β-galactosidase as a reporter. GFP was also used as a reporter for the expression of *fyuA* [7] and *hemR* [8], for the translocation of the YopE protein [9] in *Y. enterocolitica*, and for the expression of *yopE* in *Y. pseudotuberculosis* [10]. These examples demonstrate that reporter systems can be used to characterize the mechanism of virulence in *Yersinia*; however, to date there have been no large-scale efforts of this nature.

Of particular interest to the present *Y. pestis* study, and as another example of real-time characterization of gene expression in bacteria, a GFP reporter system was used to study the production of flagella in *E. coli*. [11] A panel of reporter clones bearing *gfp* under the control of promoters for flagella related genes was developed to investigate the cascade of events leading to flagella production. Interestingly, the Type III secretion system is thought to have evolved from the flagellar system. [12] This, along with the earlier reporter studies in *Yersinia*, supports the

use of a GFP reporter system to characterize the regulation of virulence and the Type III secretion mechanism in *Y. pestis*.

Here, in order to gain a better understanding of the changes in the expression of virulence factors associated with thermal induction in *Y. pestis*, the expression of six Type III secretion components was monitored in real time using GFP as a reporter. The six genes, *yopE, sycE, lcrE (yopN), yopK, yopT,* and *yscN*, which are located on the pCD1 plasmid of *Y. pestis*, were determined to be differentially expressed in response to the temperature shift associated with the change from the physiological environment of the flea to that of the mammalian host, i.e. 26°C to 37°C. Results presented here demonstrate the ability to monitor real-time induction of *Y. pestis* virulence factor genes *in vivo,* and lay the foundation for future studies aimed at defining environmental and host factors that may contribute to virulence, and ultimately, to the high lethality of *Y. pestis*.

Materials and Methods

General

The GFP plasmid pEGFP was obtained from Clontech (Mountain View, CA). All media used for the culture of clones bearing derivatives of pEGFP vector were supplemented with carbenicillin to 50 μg/mL. *Y. pestis* non-pigmented strain KIM D27 was obtained as a gift from Dr. Vladimir Motin (Univ. Texas Medical Branch, Galveston, TX) and from the laboratory of Dr. Emilio Garcia (Lawrence Livermore National Laboratory). WCS media was prepared according to the procedure of Fowler and Brubaker [3] with the modifications that the concentration of glycine was 52 mM and the concentration of potassium D-gluconate was 40 mM.

Creation of reporter plasmids

The intergenic regions of three pairs of bicistronic genes (*yop*E/*syc*E, *yop*K/*yop*T, and *ysc*N/*lcr*E) on the pCD1 plasmid of *Y. pestis* were amplified using *Pfu* polymerase, and purified on spin columns (QIAgen). The primers were designed to include the first 12-26 amino acids of the gene on either side of the intergenic region, and to maintain the correct reading frame (primer information, Table 1). The pEGFP plasmid (Clontech) was treated with *Pvu*II and *Sma*I restriction enzymes (sequentially) to remove the lac promoter, and then with shrimp alkaline phosphatase (Stratagene). The amplified promoter regions of *Y. pestis* were inserted into the linearized vector using T4 DNA ligase (Stratagene).

Screening fluorescent clones

Competent *Yersinia pestis* KIM D27 cells were transformed with the ligation mixture by electroporation [13]. Transformed cells were plated on TBA agar with 50 μg/mL carbenicillin, and incubated at 26°C for 2 days. Individual wells of a low-fluorescent background 96-well plate (Perkin Elmer) containing 200 μL WCS media were inoculated with single colonies and incubated with shaking at 26°C for 24 h, and the temperature was then raised to 37°C. Clones from cultures that exhibited fluorescence after the temperature shift to 37ºC were sequenced for confirmation of the plasmid insert (Sequetech, Mountain View, CA).

Verification of the reporter constructs

Each *Y. pestis* clone with a confirmed pEGFP plasmid sequence was further verified by a PCR amplification of a chromosomal marker (*katY*), two markers on plasmids pCD1 and pMT1, as well as a marker from the *gfp* plasmid (primer sequences, Table 1). In addition, gel electrophoresis was used to verify that the *Y. pestis* clones carried the full complement of plasmids including pCD1, pMT1, pPCP1, and pEGFP. The clones were also tested for restricted growth in WCS media upon shift from 26ºC to 37ºC [3].

Fluorescence measurements

Stationary phase cultures of *Y. pestis* KIM D27 reporter clones in WCS media were collected by centrifugation (5000 g, 10 min, 4°C), washed three times with WCS media, resuspended in fresh WCS media to a cell density in the range of 10^7 and 10^8 cfu/mL, and aliquoted into a 96-well plate (200 μL/well). These cultures were covered with mineral oil (50 μL/well), incubated with shaking at 26°C for 16 h and then the temperature was raised to 37°C.

The assay involved repeated cycles of shaking (1mm orbital, normal speed, 30 min) and photometry and fluorescence measurements in a Victor2 plate reader (Perkin Elmer). Cell densities were calculated empirically from the optical density measurements (600 nm filter, 10 nm bandwidth, 0.1 s integration). Fluorescence measurements (485 nm excitation, 510 and 580 nm emission, 10 nm bandwidths, 0.5 s integration times) were corrected for autofluorescence [14, 15] using an empirically determined relationship between the emission at 580 nm and 510 nm for the negative control *Y. pestis* KIM D27/pCF39B11.

Results

Clones of *Yersinia pestis* KIM D27 were constructed with the expression of the GFP gene, *gfp,* under the control of promoters for six virulence factors. The virulence factors studied here are *yopE* (in plasmid, pCFEG7), *sycE* (pCFEG14), *yopK* (pCF39A10), *yopT* (pCFKG15), *lcrE/yopN* (pCFNG6), and *yscN* (pCFNG9). The resulting plasmids, shown in parenthesis, after the promoters for the virulence factors were inserted upstream and in-frame with *gfp* are described in Table 1. Positive and negative control clones were also used with the expression of *gfp* under either the *lac* promoter (pEGFP) or no promoter (pCF39B11), respectively. PCR primers were designed to amplify the intergenic regions and 50 to 66 flanking bases of the three pairs of bicistronic genes, *sycE*/*yopE*, *yopK*/*yopT*, and *lcrE*/*yscN* (Table 1). The reporter plasmids were transformed into *Y. pestis* strain KIM D27 by electroporation [13], and then screened for thermal induction of fluorescence. Plasmids from selected fluorescent clones were sequenced (data not shown) to verify the correct promoter sequence.

Several steps were taken to ensure that the introduction of the high copy number pEGFP-based plasmids did not interfere with the *Y. pestis* plasmids or with the reporter assays. The maintenance of the three native *Y. pestis* plasmids [16] pMT1 (101 kb), pCD1 (70 kb) and pPCP1 (9.5 kb), as well as the *gfp*-bearing plasmid pEGFP (3.5 kb), was determined by agarose gel electrophoresis and confirmed using PCR (see Table 1 for primer details). The low-calcium response phenotype of *Y. pestis* in WCS media [3] was also verified for each clone. Briefly, *Y. pestis* ceased growth in this media upon temperature transition from 26ºC to 37ºC unless the media was supplemented with calcium. The recovery of the growth with calcium

supplementation is shown in Figure 1 for the positive control, *lac-gfp* promoter fusion (*Y. pestis* KIM D27 with plasmid pEGFP).

Real-time reporter experiments involved incubating *Y. pestis* with the reporter clones in 200 µL of WCS media in 96 well format, first at 26ºC with temperature shift to 37ºC, and monitoring increase in fluorescence as a measure of gene induction. Plots of fluorescence intensity over time were determined using averaged data from triplicate wells. Representative plots are shown in Figure 2 for the *yopE* and *yopK* reporters. Analysis of these plots for 6-10 biological replicates for each clone yielded the basal expression levels (before the temperature transition) and the rates of induction (after the temperature transition), both without calcium supplementation and with calcium supplementation (see Table 2). The results of these two types of data analyses are discussed in turn.

The basal expression level at 26ºC was determined for each of the *Y. pestis* clones in media supplemented to 4 mM calcium chloride. The positive control with the expression of *gfp* under the *lac* promoter had the highest level of basal expression. The basal level for the clones with *gfp* under the control of the *yopE* and *sycE* promoters was 15% of the basal expression level for the positive control. Data for the other clones are collected in Table 2. The basal expression levels of the reporter clones were not significantly different when the media was not supplemented with calcium (Table 2).

The average rates of the thermal induction for each of the *Y. pestis* virulence factors, for 6-10 biological replicates, are shown in Table 2. The *yopE* reporter was found to display the highest

degree of thermal induction, followed by the *yopK* promoter. The presence of calcium in the media repressed the thermal induction resulting in lower rates of induction (see Figure 1 and Table 2). The ratios of the initial rates of induction without added calcium compared to with calcium supplementation are included in Table 2.

Discussion

Promoters for virulence factors of *Yersinia pestis* were fused to the gene for green fluorescent protein (*gfp*) to create the real-time reporters. In order to achieve optimal signal-to-noise, we chose the high copy number, enhanced GFP pUC based plasmid, pEGFP. One concern with this choice was that the system would suffer from promoter dilution due to the high copy number and therefore insufficient transcription factors for induction of expression. However, comparing our results with the microarray data of Motin and Garcia [17] suggested that the promoter dilution effect was not significant. Notably, the microarray studies where performed with *Y. pestis* grown in BCS media [3] and the real-time studies reported here were performed in WCS media, which results in restricted growth (Figure 1). However, our results correlate well with the microarray data. The largest difference between the two systems was seen for *yopE*, which in our system demonstrated an 11-fold ratio for no-calcium compared to with-calcium, whereas the microarray results showed a 19-fold ratio. These values are not significantly different considering the standard deviations in the measured rates of induction (Table 2).

Two aspects of the expression of virulence factors in *Y. pestis* were revealed in this study: the basal levels of expression at 26ºC and the rates of induction after temperature shift to 37ºC. The basal levels of expression measured for the six promoters in this study range from 0.8% to 15% of the basal level measured for the positive control, *lac-gfp* promoter fusion. Expressed in other terms, there was almost a twenty-fold difference in the basal levels of expression. It should be noted that protein levels of virulence factors in *Y. pestis* may not necessarily follow this same trend, as the rates of proteolysis of the proteins may be quite different than that of the GFP

reporter. Further proteomic studies are required to address how proteolysis rates of GFP and *Y. pestis* virulence factors may affect the correlation of protein levels with gene expression levels.

We also observed that the principle of proportional gene expression dynamics, which states that gene expression changes are proportional to their basal expression levels, was not conserved in this real-time system [18]. That is, that the basal levels of gene expression were not reflected in the level of induction. It should be noted that this principle was developed for genome wide expression results and may not be entirely applicable to the present study where six genes were studied; however, the data for *sycE* and *yscN* reveal the discrepancies in the initial basal level expression and the subsequent level of induction. In the case of *sycE*, which had a relatively high basal level of expression, the level of induction was much less than that of *yopE*, which had a similarly high level of basal expression. In the case of *yscN*, the relatively low level of basal expression did not correlate with a low level of induction. These observations could be interpreted as either an indication that the basal levels of expression are more tightly regulated than predicted, or that the level of induction is not as tightly linked to the basal levels of expression as expected. It is not clear at this point whether this is a general observation for a biological system that is optimized for two very different physiological environments (flea vector versus mammalian host), or whether the regulation of gene expression in this system is more complex than the systems used to develop the principle.

Also of note, the standard deviations in the initial rates of induction measured in this study were significant, for example, the rate of the induction of *yopK* without calcium had a 50% standard deviation (see Table 2). The magnitudes of the standard deviations in the rate of

induction suggest that some regulatory element of control for these genes was not adequately constrained in our experiments. One explanation for this phenomenon is that quorum sensing may be involved in the regulation of virulence factor induction. While quorum sensing has not been previously demonstrated in *Y. pestis*, our preliminary data (manuscript in preparation) support this theory. In addition, our theory is further supported by the observation that the genome of *Y. pestis* [16, 19] possesses two homologues of the *luxI* autoinducer synthase as well as two homologues of the *luxR* transcriptional repressor, responsible for quorum sensing in other organisms [20]. Notably, quorum sensing has been observed to play a role in the regulation of virulence factor expression in *Y. pseudotuberculosis* [21] and *Y. enterocolitica* [22]; however, quorum sensing was previously reported not to exist in *Y. pestis* [23]. This report was the result of a functional mutation study of only one of the quorum sensing homologues in the *Y. pestis* genome, and thus may not have fully addressed the existence of quorum sensing due to the subsequent discovery of the second quorum sensing homologue.

Further work to determine the effect of quorum sensing on the regulation of virulence factors in *Y. pestis* is underway and may uncover an as yet unidentified regulatory element of virulence. In addition to quorum sensing, the *Y. pestis* real-time system reported is also of interest for characterization of other environmental and host elements that may regulate virulence in *Y. pestis*. In conclusion, results presented here demonstrate the ability to study expression of virulence factors in real time, in living cells, with the potential for in-depth characterization of the mechanisms of virulence and regulatory networks that contribute to *Y. pestis* virulence and ultimately to the high lethality of this human pathogen.

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Figure Legends

Figure 1. The restricted growth phenotype in WCS media was observed for all *Yersinia pestis* reporter clones used in this study. The growth response curve for *Y. pestis* KIM D27/pEGFP is shown at four calcium concentrations (supplementation to 0, 2, 4, and 8 mM). Cultures were incubated at 26ºC and then shifted to 37ºC at time 0h. Cell densities were calculated from the measured optical densities at 600 nm.

Figure 2. The expression of the *yopE* and *yopK* genes in *Yersinia pestis* increased following the temperature shift from 26ºC to37ºC at time 0h, in a calcium-regulated manner, as measured by the fluorescence intensity per colony forming unit. Fluorescence from GFP was monitored at 510 nm using an excitation centered at 485 nm (10 nm bandpass filter). Curves represent *Y. pestis* KIM D27/pCFEG7 either with 4 mM calcium supplementation (filled circles) or without calcium supplementation (open circles), and *Y. pestis* KIM D27/pCF39A10 either with 4 mM calcium supplementation (filled squares) or without calcium supplementation (open squares). Other *Y. pestis* reporters showed similar fluorescence increases as a function of temperature shift.

Table 1. PCR/Sequencing Primers, Plasmids, and *Y. pestis* Strains

Amplified DNA Region	Primer Sequence
sycE/yopE	5'-CTGACACAGATGTCGGCAGG-3'
	5'-ACGAAAGTTGTTGAAATAATTGAGTG-3'
yopK/yopT	5'-ATAGTTGAATATGGTAGTGTCCGTG-3'
	5'-CAGGAGCCGACTGTTCAAG-3'
lcrE/yscN	5'-TGCCATGACGAATATGATGAG-3'
	5'-GCGGGGTATTGCCATAAG-3'
pCD1 (*yopE*)	5'-TTGAGTGATAGCTTGTTCAAATG-3'
	5'-GCAGGGGCAGTGATGTAGA-3'
pMT1 (ORF38)	5'-AAGAGGGTCGCAGCTATGTC-3'
	5'-CAACCACACGAATGGTTTTAC-3'
pEGFP	5'-AATACGCAAACCGCCTCTC-3'
	5'-CTCCTCGCCCTTGCTCAC-3'
katY	5'-TCAATCTAATAATACAAACGCCCTAC-3'
	5'-GCGGAGAGTTATTAGTACGGGTAAG-3'

Plasmid	Description
pEGFP	pUC-based vector, *gfp* under the control of the *lac* promoter (Clontech)
pCF39B11	pEGFP Δ*Sma*I-*Pvu*II (promoterless *gfp*)
pCFEG7	pEGFP-based vector, *gfp* under the control of the *yopE* promoter
pCFEG14	pEGFP-based vector, *gfp* under the control of the *sycE* promoter
pCF39A10	pEGFP-based vector, *gfp* under the control of the *yopK* promoter
pCFKG15	pEGFP-based vector, *gfp* under the control of the *yopT* promoter
pCFNG6	pEGFP-based vector, *gfp* under the control of the *yscN* promoter
pCFNG9	pEGFP-based vector, *gfp* under the control of the *lcrE* promoter

Strain	Description
Y. pestis KIM D27	*pgm*$^-$ biovar Mediaevalis [3]
Y. pestis KIM D27/pEGFP	*Y. pestis* KIM D27 transformed with pEGFP
Y. pestis KIM D27/pCF39B11	*Y. pestis* KIM D27 transformed with pCF39B11
Y. pestis KIM D27/pCFEG7	*Y. pestis* KIM D27 transformed with pCFEG7
Y. pestis KIM D27/pCFEG14	*Y. pestis* KIM D27 transformed with pCFEG14
Y. pestis KIM D27/pCF39A10	*Y. pestis* KIM D27 transformed with pCF39A10
Y. pestis KIM D27/pCFKG15	*Y. pestis* KIM D27 transformed with pCFKG15
Y. pestis KIM D27/pCFNG6	*Y. pestis* KIM D27 transformed with pCFNG6
Y. pestis KIM D27/pCFNG9	*Y. pestis* KIM D27 transformed with pCFNG9

Table 2. Real-Time Data for *Y. pestis* Virulence Factors

plasmid	promoter	*n*	initial rate (-Ca) ($cfu^{-1}h^{-1}$)	σ[a]	initial rate (+Ca) ($cfu^{-1}h^{-1}$)	σ[a]	rate ratio	σ[a]	basal rate (%)[b]	σ[a]
pEGFP	*lac*	6	3	1	1.9	0.9	1.6	0.8	100	36
pCF39B11	none	10	0.001	0.002	0.001	0.002	1	4	0.0	0.5
pCFEG7	*yopE*	9	7	5	0.8	0.4	11	7	15	7
pCFEG14	*sycE*	9	0.6	0.2	0.5	0.3	1	1	15	5
pCF39A10	*yopK*	7	4	2	0.9	0.8	6	3	13	3
pCFKG15	*yopT*	10	0.12	0.05	0.10	0.07	2	2	4	1
pCFNG6	*yscN*	9	0.19	0.09	0.06	0.06	7	6	0.8	0.6
pCFNG9	*lcrE*	10	0.19	0.09	0.06	0.06	3	2	3.3	0.6

[a] standard deviation in the measured value for the *n* replicates

[b] percentage of that measured for the positive control with *lac* promoter